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The Contribution of Interleukin-12A Genotypes to Oral Cancer Risk in Taiwanese

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Abstract. *Background/Aim: Oral cancer incidence is highest worldwide in Taiwan, and practical markers for personalized therapeutic strategies such as immunotherapies, is lacking. Interleukin-12 (IL12) is a cytokine that is reported to exhibit potent tumoricidal effects, however, its genotypic contribution to oral cancer is still largely unknown. We aimed to examine whether IL12A rs568408 and rs2243115 genotypes are associated with oral cancer risk in Taiwan. Materials and Methods: Genotypic characteristics of IL12A were determined among 958 oral cancer cases and age- and gender-matched individuals via typical polymerase chain reaction–restriction fragment length polymorphism methodology. Results: The variant genotypes of IL12A rs568408 and rs2243115 were not found to be significantly associated with elevated oral cancer risk (all p>0.05). Moreover, there was no interaction between IL12A genotypes and personal smoking, alcohol drinking and betel quid chewing behaviors (all p>0.05). Conclusion: IL12A rs568408 and rs2243115 genotypes may not serve as good predictors for oral cancer risk.*

According to the 2017 Statistics of Causes of Death published by the Ministry of Health and Welfare, oral cancer is the fourth most common cancer in males and the fourth leading cause of cancer death in males in Taiwan, where the incidence of oral cancer is the highest in the world (1-3). Epidemiological studies have shown that the development and susceptibility to oral cancer for Taiwanese is determined by subtle genomic variations [such as single nucleotide polymorphisms (SNPs)] and several lifestyle factors (such as alcohol/tobacco/betel quid consumption, poor oral hygiene habits) and viral infectious status (4-7). Thus, a better target or marker for advanced personalized therapeutic approaches such as immunotherapies are an urgent need. To fulfill this aim, mounting studies have provided evidence for that specific genotypes are associated with increased oral cancer risk in cigarette smokers (8, 9), and betel quid chewers (10, 11) using genotyping. These findings are extremely valuable in elucidating the contribution of genomic, environmental and personal behavioral aspects to oral cancer development and providing a better consulting etiology for therapeutic decision making and genomic pharmacology for each patient with oral cancer.

Interleukin 12 (IL12) is a multi-functional cytokine originally identified as stimulatory factor for natural killer cells and maturation factor for lymphocytes (12, 13). IL12 has been found to stimulate the proliferation and cytolytic capacity of natural killer cells, and activate their capacity for cytokine production, particularly interferon-γ (IFNγ) (14, 15). Furthermore, IL12 has been reported to serve in bridging innate and adaptive immunity by promoting the differentiation of T-helper 1 (Th1) cells (16, 17). In animal model experiments, IL12 has been found to have antitumor capacity since mice lacking IL12 subunit p35 had earlier and more papilloma development compared with wild-type mice (18). Moreover, mice deficient in IL12 receptor chain (IL12Rβ2) had a faster growth of B16 melanomas than did

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wild-type ones (19). Lastly, exogenous administration of IL12 significantly suppressed the growth of sarcoma, melanoma, lung carcinoma and breast carcinoma in transplanted mouse models (20-22). Most interesting, although IL12 has certain side-effects, its curative effect is impressive and clinically significant. For instance, phase I/II trials of IL12 in patients with B-cell lymphoma or Kaposi sarcoma were both very successful in 2002 and 2007 (23, 24). IL12 treatment can have synergistic effects with IL18 in restoring the intratumoral natural killer cell functions in major histocompatibility complex class I-deficient tumors (23, 24). Combined with natural killer cell-secreted IFNγ, IL12 can inhibit tumor angiogenesis and suppress tumor growth (25).

Human *IL12A* and *IL12B* genes are located at chromosomes 3 and 5, respectively. Among the SNPs of *IL12A*, rs568408 is the polymorphic site most frequently examined. *IL12A* rs568408 is located in the 3'-untranslated region (UTR) and may influence the level of IL12 production (26, 27). Another polymorphic site, *IL12A* rs2243115, is situated in the 5'UTR, and its functional significance has not been well examined or conclusively reported. In the current study, we aimed to investigate the contribution of *IL12A* genotypes to the risk of oral cancer and to examine the joint effect of personal behavioral habits and *IL12A* genotypes on oral cancer risk in Taiwan.

Materials and Methods

Population sampling methodology. Nine hundred and fifty-eight patients diagnosed with oral cancer were recruited with help from the cancer team at the Outpatient Clinics of General Surgery, collecting the clinical characteristics of patients, including histological details, which were all graded and defined by experts in pathology. Patients with history of any other cancer were excluded from the databank, and all participants were Taiwanese and voluntarily completed a self-administered questionnaire and provided their blood sample for genotyping studies. Some number of healthy volunteers as controls were selected by matching for age, gender, and behavioral factors alcohol, tobacco and areca-nut consumption after initial random sampling from the Health Examination Cohort of the China Medical University Hospital. The exclusion criteria of the controls included previous malignancy, metastasized cancer from other or unknown origin, and any genetic or familial disease. The study was approved by the Institutional Review Board of the China Medical University Hospital (DMR101-IRB1-306) and written informed consent was obtained from all participants. Several selected characteristics of all the investigated participants are summarized in Table I.

IL12A polymerase chain reaction (PCR)-restriction fragment length polymorphism genotyping conditions. Genomic DNA from the peripheral blood leucocytes of each patient with oral cancer and control was prepared using the QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan, ROC) (28, 29) and further processed in typical PCR processes as previously published (30, 31). For *IL12A* rs2243115 and rs568408, the forward primers introduce a mismatched A to replace C and a mismatched T to replace C, respectively, at -3 bp from the polymorphic sites to create *BstNI*- and *NdeI*- (New England BioLabs, Ipswich, MA, USA) digestible restriction sites, respectively. For *IL12A* rs568408, the primers were 5'-AGAAAAGACCTGTGAACAAAACGACT-3' (forward) and 5'-AGATGGCTCACTAGATGCCAGG-3' (reverse). For *IL12A* rs2243115, the primers were 5'-GAAGGAT GGGACYATTACATCCATAT-3' (forward) and 5'-CAGGATGGATA TTTTCCCTTCT-3' (reverse). The wild-type allele *IL12A* rs2243115T generated a full fragment of 122 bp, while the variant allele *IL12A* rs2243115G produced two fragments of 93 and 29 bp after PCR. For *IL12A* rs568408, the wild-type allele *IL12A* rs568408G produced two fragments of 98 and 23 bp and the variant allele *IL12A* rs568408A resulted in a full fragment of 121 bp.

The PCR cycling was set as: one cycle at 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s; and a final extension at 72˚C for 10 min. Agarose gel used was 3% and the electrophoresis conditions were 100 V for exactly 20 min. The genotype analysis was performed by three researchers independently and blindly. For each *IL12A* SNP, 5% of the PCR products were randomly selected for direct sequencing and the results from PCR-restriction fragment length polymorphism and direct sequencing were 100% concordant to each other.

Statistical analyses. Student's *t*-test was used to compare the difference of age between case and control groups. Pearson's chi-square test was used to compare the distribution of the *IL12A* genotypes between the two groups. The associations between the *IL12A* genotypes and cancer risk were estimated by computing odds ratios (ORs) and corresponding 95% confidence intervals (CIs) from logistic regression analysis. Any comparison result with $p<0.05$ was recognized as statistically significant.

Results

The basic and clinical data including age, gender, personal habits for the investigated 958 patients with oral cancer and the 958 matched controls are summarized and compared in Table I. In addition, the tumor sites of all the patients are also presented in Table I. Firstly, there was no difference between the case and control groups in the aspects of age and gender (p=0.3755 and >0.99, respectively) (Table I). Secondly, the proportions of smokers, alcohol drinkers and betel quid chewers were found to be higher in the oral cancer group than those in the age- and gender-matched control group (p=0.0107, 0.0377 and 0.0001, respectively), which indicates that these behavioral habits are associated with oral cancer for Taiwanese (Table I). Lastly, the most prevalent tumor sites of oral cancer were the tongue (41.4%) and buccal mucosa (37.2%) among this Taiwan oral cancer population (Table I).

The distribution of genotypic frequencies of *IL12A* rs568408 and rs2243115 among the controls and cases are summarized in Table II. Firstly, the genotypic frequencies for the two SNPs investigated fit well with the Hardy–Weinberg equilibrium for the control group (p>0.05). Secondly, there was no significant difference in the distribution of *IL12A*

Table I. *Characteristics of the investigated 958 patients with oral cancer and 958 healthy controls.*

Characteristic	Controls (n=958)	Cases (n=958)	*p*-Value
Age, years			
Mean±SD	56.8±8.7	56.4±7.5	0.3755[a]
Gender, n (%)			
Male	728 (76.0%)	728 (76.0%)	>0.99[b]
Female	230 (24.0%)	230 (24.0%)	
Personal habit, n (%)			
Cigarette smoking	668 (69.7%)	718 (74.9%)	**0.0107**[b]
Alcohol drinking	642 (67.0%)	684 (71.4%)	**0.0377**[b]
Betel quid chewing	508 (53.0%)	773 (80.7%)	**<0.0001**[b]
Primary tumor site, n (%)			
Tongue		397 (41.4%)	
Buccal mucosa		356 (37.2%)	
Mouth floor		39 (4.1%)	
Retromolar trigone		33 (3.4%)	
Alveolar ridge		29 (3.0%)	
Palate		27 (2.8%)	
Lip		39 (4.1%)	
Other		38 (4.0%)	

SD: Standard deviation; [a]Based on Student's *t*-test; [b]Based on chi-square test. Significant *p*-values ($p<0.05$) are shown in bold.

Table II. *Distribution of interleukin-12A (IL12A) rs568408 and rs2243115 genotype among 958 patients with oral cancer and 958 controls.*

IL12A genotype	Cases		Controls		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
rs568408						
GG	689	71.9%	677	70.7%	1.00 (Reference)	
AG	231	24.1%	250	26.1%	0.91 (0.74-1.12)	0.3624
AA	38	4.0%	31	3.2%	1.21 (0.74-1.96)	0.4526
AG+AA	269	28.1%	281	29.3%	0.94 (0.77-1.15)	0.5445
P_{trend}						0.4570
rs2243115						
TT	793	82.8%	809	84.4%	1.00 (Reference)	
GT	151	15.7%	138	14.4%	1.12 (0.87-1.43)	0.3897
GG	14	1.5%	11	1.2%	1.30 (0.59-2.88)	0.5190
GT+GG	165	17.2%	149	15.6%	1.13 (0.89-1.44)	0.3234
P_{trend}						0.5756

CI: Confidence intervaI; OR: odds ratio. [a]Based on chi-squared test without Yate's correction.

rs568408 genotypes between oral cancer and control groups (*p* for trend=0.4570). The frequencies of the heterozygous variant AG and homozygous variant GG of *IL12A* rs568408 were 24.1% and 4.0% in the oral cancer group and not significantly different from those in the control group (26.1% and 3.2%, respectively). Thus, neither the AA (OR=1.21, 95% CI=0.74-1.96, $p=0.4526$) nor AG (OR=0.91, 95% CI=0.74-1.12, $p=0.3624$) genotype at *IL12A* rs568408 can serve as a potential biomarker for oral cancer in Taiwanese. Moreover, considering the mere presence of the A allele by combining variant AG with AA at *IL12A* rs568408 also did not alter the risk for oral cancer risk among Taiwanese compared with the wild-type GG genotype (OR=0.94, 95% CI=0.77-1.15, $p=0.5445$; Table II). Similarly, there was no significant difference in the distribution of *IL12A* rs2243115 genotypes between oral cancer and control groups (*p* for trend=0.5756). The frequencies of the heterozygous variant GT and homozygous variant GG of *IL12A* rs2243115 were 15.7% and 1.5% for the oral cancer group, non-significantly different from those of the control group (14.4% and 1.2%, respectively). Thus, neither the GG (OR=1.30, 95% CI=0.59-2.88, $p=0.5190$) nor GT (OR=1.12, 95% CI=0.87-1.43, $p=0.3897$) genotype at *IL12A* rs2243115 can serve as a potential biomarker for Taiwan oral cancer. The presence of a G allele at *IL12A* rs2243115 also did not alter the risk for oral cancer risk among Taiwanese compared with the wild-type TT genotype (OR=1.13, 95% CI=0.89-1.44, $p=0.3234$) (Table II).

The distribution of allelic frequencies of *IL12A* rs568408 and rs2243115 are summarized in Table III. Consistent with the findings in Table II, the presence of allele A at *IL12A* rs568408 was not associated with an altered risk of oral cancer compared with allele G ($p=0.8263$). Similarly, the variant G allele of *IL12A* rs2243115 was not associated with any significantly altered risk for oral cancer ($p=0.2798$; Table III).

Since betel quid chewing behavior and those of cigarette smoking and alcohol drinking may contribute to the etiology of oral cancer, we were also interested in the interaction of the genotype of *IL12A* with these personal behaviors on the determining oral cancer susceptibility. The joint effects of *IL12A* rs568408 with betel quid chewing status are presented in Table IV. In the Table, patients with oral cancer and matched controls were stratified according to their betel quid chewing status and ORs were analyzed. The results show that non-betel quid chewers carrying the variant genotypes at *IL12A* rs568408 did not have altered risk of oral cancer ($p=0.8930$). Similarly, there was no significantly elevated oral cancer risk for ever chewers with variant genotypes at *IL12A* rs568408 ($p=0.5802$). There were also no significant findings for variants of *IL12A* rs2243115 (Table V). Furthermore, we found no significant interaction between these *IL12A* genotypes and cigarette smoking status nor alcohol drinking status for oral cancer risk (data not shown).

Discussion

In the current study, the contributions of *IL12A* rs568408 and rs2243115 genotypes to oral cancer susceptibility were investigated for the first time in a Taiwanese population. From

Table III. *Distributions of interleukin-12A (IL12A) rs568408 and rs2243115 allelic frequencies among the 958 patients with oral cancer and the 958 controls.*

	Cases		Controls		OR (95% CI)	*p*-Value[a]
SNP	n	%	n	%		
rs568408						
G	1,609	84.0%	1604	83.7%	1.00 (reference)	
A	307	16.0%	312	16.3%	0.98 (0.83-1.17)	0.8263
rs2243115						
T	1,737	90.7%	1,756	91.6%	1.00 (reference)	
G	179	9.3%	160	8.4%	1.13 (0.90-1.41)	0.2798

CI: Confidence intervaI; OR: odds ratio; SNP: single nucleotide polymorphism. [a]Based on chi-squared test without Yate's correction.

Table IV. *Distribution of interleukin-12A (IL12A) rs568408 genotypes among the 958 patients with oral cancer and the 958 controls after stratification by betel quid chewing behavior.*

Betel quid behavior	*IL12A* rs568408 genotype, n (%)			*p*-Value[a]
	TT	GT	GG	
Non-chewers				
Controls	316 (70.2%)	119 (26.4%)	15 (3.3%)	
Cases	132 (71.3%)	46 (24.9%)	7 (3.8%)	0.8930
Chewers				
Controls	361 (71.1%)	131 (25.8%)	16 (3.1%)	
Cases	557 (72.1%)	185 (23.9%)	31 (4.0%)	0.5802

[a]Based on chi-squared test without Yate's correction.

Table V. *Distribution of interleukin-12A (IL12A) rs2243115 genotypes among the 958 oral cancer patients and the 958 controls after stratification by betel quid chewing behavior.*

Betel quid behavior	*IL12A* rs2243115 genotype, n (%)			*p*-Value[a]
	GG	AG	AA	
Non-chewers				
Controls	376 (83.6%)	68 (15.1%)	6 (1.3%)	
Cases	151 (81.6%)	29 (15.7%)	5 (2.7%)	0.4710
Chewers				
Controls	433 (85.2%)	70 (13.8%)	5 (1.0%)	
Cases	642 (83.0%)	122 (15.8%)	9 (1.2%)	0.5811

[a]Based on chi-squared test without Yate's correction.

the genotyping results, neither *IL12A* rs568408 nor rs2243115 were found to be suitable genomic biomarkers for detection and prediction of oral cancer risk among Taiwanese (Table II). The findings were further validated by the allelic frequency analysis revealing neither of the variant alleles at *IL12A* rs568408 and rs2243115 were associated with altered oral cancer risk (Table III). These findings support the idea that any involvement of *IL12* gene and its protein in the carcinogenesis of oral cancer may simply not be critical at *IL12A* rs568408 and rs2243115.

The genotypes of pro-inflammatory cytokine gene *IL12A* are reported to be associated with many human diseases. For instance, The AA genotype of *IL12A* rs568408 is associated with higher risk of Graves' disease in Chinese populations (32). The variant AA genotype at *IL12A* rs568408 is also associated with higher risk of asthma in Taiwan (33). On the other hand, Sima and colleagues found that variant G allele carriers of *IL12A* rs2243115 were at higher risk of brain tumors compared with those carrying the wild-type T allele (34). Almost at the same time, Wang and colleagues provided evidence that the variant G allele at *IL12A* rs2243115 also contributes to higher risk of chronic obstructive pulmonary disease (35). To our knowledge, there is no other report examining the contribution of *IL12A* genotypes to oral cancer. Interestingly, a higher A allele frequency was found among patients with oral lichen planus than controls in an East Chinese cohort study (36). Although it is reported by a meta-analysis that the genotypes of *IL12A* rs568408 were found to significantly associated with overall cancer risk, especially among Asian ethnicities (37), in the current study, we did not find any association between *IL12A* rs568408 or rs2243115 genotypes with oral cancer risk in Taiwanese.

We have to take into consideration that the phenotype of IL12 is determined not only by the genotype of *IL12A* but also by that of *IL12B*. Frequently, the genotype of *IL12B* seems to play a more role critical in determining the serum level of IL12. For instance, the serum levels of IL12 were found to be much higher in patients with type 1 diabetes with *IL12B* rs3212227 AA genotype than those with AC or CC genotypes (38, 39). On the contrary, the levels of IL12 were reported to be higher in peripheral blood mononuclear cells from individuals with CC genotype at *IL12B* rs3212227 than those with AC or AA genotypes, upon stimulation with lipopolysaccharide (40). Furthermore, IL12A and IL12B may control the secretion of each other. It has been shown that the presence of the variant genotype *IL12B* rs3212227 correlated with increased secretion of IL12A but not of IL12B itself (39). In addition, the combinatory effects of *IL12A* and *IL12B* need to be examined. In 2019, although the we found the genotypes of *IL12B* were not associated with altered oral cancer risk, the *IL12B* rs3212227 genotype was significantly associated with oral cancer risk specifically for betel quid chewers but not non-chewers (40). There is no synergistic effect for either *IL12A* rs568408 or rs2243115 with *IL12B* rs3212227 on determination of oral cancer risk (data not shown).

In this study, the interaction analysis of the genotype of *IL12A* at rs568408 and rs2243115 and the major personal

behavioral risk factors for Taiwan oral cancer were evaluated. The results showed that those people with variant allele at *IL12A* rs568408 (Table IV) or rs2243115 (Table V) with or without betel quid chewing habit had similar susceptibility to oral cancer. No genotype–habit interaction was found among cigarette smokers or alcohol consumers either (data not shown). In the present research, we did not find any genotype–phenotype correlation among the investigated Taiwanese individuals. Further studies using primarily cultured cells from tumor sites of patients with oral cancer are strongly recommended to explore the effects of betel quid compounds on primarily cultured cells with different *IL12A* rs568408, *IL12A* rs2243115 and *IL12B* rs3212227 genotypes in order to validate the genotype–phenotype correlation of these SNPs.

In conclusion, the study indicated that genotypes of *IL12A* rs568408 and rs2243115 are not determiners of oral cancer risk among Taiwanese. The findings should be validated in more populations in similar or different ethics.

Conflicts of Interest

The Authors declare no conflicts of interest with any company or person.

Authors' Contributions

Research design was performed by LCH, SLC. Patient and questionnaire summaries were provided by SLC and HCL. Experimental work was carried out by WYC and CWS. Statistical analysis was performed by LCY and LHT. LCY, TCW, and BDT wrote the article; BDT, CWS, and TCW reviewed it and are responsible for the revision.

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